PATENT APPLICATION

#14

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

TAKAYAMA, HIROAKI, et al.

Appln. No.: 09/214,155 Group Art Unit: 1616

Filed: December 29, 1998 Examiner: QAZI, S

For: VITAMIN D_3 DERIVATIVE AND ITS PRODUCTION METHOD

SUBMISSION OF EXECUTED SUPPLEMENTAL DECLARATION UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Submitted herewith is an executed Supplemental Declaration Under 37 C.F.R. §1.132 signed by Seiichi ISHIZUKA.

Respectfully submitted,

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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Hiroaki TAKAYAMA, et al.

Appln. No.: 09/214,155 Group Art Unit: 1616

Filed: December 29,1998 Examiner: Sabiha N. Qazi

For: VITAMIN D3 DERIVATIVE AND ITS PRODUCTION METHOD

SUPPLEMENTAL DECLARATION UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Seiichi Ishizuka, hereby declare and state:

THAT I am a citizen of Japan;

THAT I have received a Masters Degree in 1974 and a Ph.D. degree in 1996 from Tohoku University;

THAT I am a member of several Japanese and American Scientific Societies related to research in vitamin D and bone and mineral metabolism, a referee for two scientific journals (Biochemistry and J. Nutritional Biochemistry), and the recipient of two scientific research awards (Vitamin D research award from Brown University (1995) and scientific research award from Japanese Society for Bone and Mineral Research (1997)).

THAT I have been employed by Teijin Institute for Bio-Medical Research since 1975, in the Department of Bone and Calcium Metabolism, where I have been involved in the study of the metabolism, biological activities and mechanism of actions of Vitamin D_3.

I have thorough knowledge of the invention in the above-referenced patent application and I have read the final Office Action of March 1, 2000 issued in reference to the application. In response to the final Office Action, I submit herewith this Supplemental Declaration comparing the potency of 20S-forms versus 20R-forms of Vitamin D derivatives and as evidence that the 20S forms are significantly more potent than the 20R forms in their ability to induce cellular differentiation.

Comparison of Activity for 20S- versus 20R-forms of Vitamin D derivatives for induction of HL-60 cells to differentiate.

Materials and methods.

HL-60 cells were purchased from a cell bank (Japanese Cancer Research Resource Bank, Cell Number: JCRB 0085), and stored as a frozen stock to prevent any changes from occurring in the cell characteristics attributable to successive cultivation. Before the initiation of experiments, the cells were thawed, and passed by culturing. Cells which had been treated by successive culturing for one to six months, were used in the experiments. The successive culturing was carried out by culturing the cells in suspension, collecting the cell pellet by centrifugation, and diluting the cell pellet in fresh culture medium at a ratio of about 1/100 (1-2 x 10^5 cells/ml). The culture medium was RPMI-1640 containing 10% fetal bovine serum. Successively cultured cells were collected by centrifugation, and then were dispersed in culture medium at a concentration of 2 x 10^4 cells/ml. The suspended cells were seeded into a 24-well culture dish at 1 ml/well.

An ethanol solution containing the 20S form compounds, 68, 71, 72 or 74, at concentrations ranging from $1x10^{-9}$ M to $1x10^{-6}$ M was added to each well at 1 μl/well. Further, regarding 1 α, $25(OH)_2D_3$, an ethanol solution containing 1×10^{-7} M to 1×10^{-4} M of the compound was added at 1 μl/well, and for the control, ethanol alone was added at 1 μl/well.

An ethanol solution containing the 20R form compounds, 3, 4, 6 or 65, at concentrations ranging from $1x10^{-7}$ M to $1x10^{-3}$ M was added to each well at 1 μl/well. For the control, ethanol alone was added at 1 μl/well.

After culturing at 37°C for 4 days under a 5% CO_2 atmosphere, the cells were collected by centrifugation. Nitroblue tetrazolium (NBT) reduction activity was determined as follows: collected cells were suspended in a fresh culture medium, to which NBT and 12-O-tetradecanoylphorbol-13-acetate were added, so that the final concentrations were 0.1% and 100 nM, respectively. After mixing, the suspension was incubated at 37°C for 25 min, and the sample was removed for a cytospin centrifugation. After air drying, the cell pellet was stained with Kernschtrot, and the ratio of blue stained to unstained cells (i.e., cells showing NBT reduction) was determined under an optical microscope.

The results are shown in the following table.

Sample	Concentration (M)	% cells showing nitroblue tetrazolium activity	**Sample**	Concentration (M)	% cells showing nitroblue tetrazolium activity
Control		1.5	Control		5.8
1α,25-$(OH)_2D_3$	10^{-10} 10^{-9} 10^{-8} 10^{-7}	4.3±1.2 36.8±2.0 86.1±2.6 96.5±1.0	-	-	-
20S form			**20R form**		
Compound (68)[1]	10^{-12} 10^{-11} 10^{-10} 10^{-9}	1.7±0.3 2.8±0.7 57.7±5.0 95.7±1.0	Compound (65)[2]	10^{-8} 10^{-7} 10^{-6}	6.8 65.8 88.3
Compound (71)[3]	10^{-12} 10^{-11} 10^{-10} 10^{-9}	1.5±0.8 1.8±0.8 2.0±1.0 40.5±1.8	Compound (3)[4]	10^{-8} 10^{-7} $3x10^{-7}$	6.8 11.4 80.9
Compound (74)[5]	10^{-12} 10^{-11} 10^{-10} 10^{-9}	6.4±1.1 17.0±2.3 16.7±1.1 96.4±1.4	Compound (6)[6]	10^{-9} 10^{-8} 10^{-7} 10^{-6}	5.2 17.0 71.2 82.5
Compound (72)[7]	10^{-12} 10^{-11} 10^{-10} 10^{-9}	3.7±0.8 94.4±1.8 95.7±2.3 96.2±2.0	Compound (4)[8]	10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6}	8.1 27.9 80.0 88.7 94.5

[1] Example 2, page 33 of the specification.

[2] Page 40, line 1 of the specification.

[3] Example 5, page 36 of the specification.

[4] Page 39, line 30 of the specification.

[5] Example 7, page 37 of the specification.

[6] Page 39, line 33 of the specification.

[7] Example 1, page 32 of the specification.

[8] Page 39, line 31 of the specification.

Conclusions.

In a colorimetric cell assay which measures the ability of the compounds to induce differentiation of the HL-60 cell line vis-à-vis the reduction of nitroblue tetrazolium, the instant 20S-forms show excellent efficacy compared to the 20R-forms. Comparison of compound (68) with compound (65); compound (71) with compound (3); compound (74) with compound (6) and compound (72) with compound (4) reveals that the 20S-forms are substantially more potent, i.e., require logarithmically lower concentrations, in their ability to induce cell differentiation.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: May 9, 2000

Seiichi Ishizuka

Seiichi Ishizuka